

✦ Original research

Retinal cell death is primarily caspase-dependent in vitro and in a mouse model of chloroquine-induced acute retinal toxicity

Dhanesh Amarnani,^{1‡} Eric J. Kim,^{1‡} Brandon V. Duffy,¹ Leslie Ramos,¹ and Leo A. Kim^{1*}

1. Schepens Eye Research Institute of Mass Eye and Ear and the Department of Ophthalmology at Harvard Medical School, Boston, Massachusetts 02114, USA.

* **Corresponding author:** Leo A. Kim, 20 Staniford Street, Boston, MA 02114, USA. Contact: 323-317-8383. Email: leo_kim@meei.harvard.edu

‡ Authors contributed equally to this work

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Abstract

Purpose: To investigate the mechanism of chloroquine-induced cell death in vitro in adult human retinal pigmented epithelial (ARPE-19) cells and murine 661W photoreceptor-derived cells and to evaluate a mouse model of acute chloroquine-induced retinal toxicity.

Methods: Human RPE cells and photoreceptor cells were cultured in vitro and treated with chloroquine; cell death was measured by lactate dehydrogenase release. Lysosomal-associated membrane protein-1 (LAMP-1) staining assessed lysosomal destabilization upstream of cell death initiation. The role of cathepsins, lysosomal proteases was examined by blocking their activity. Cell permeable caspase, and RIP1 kinase inhibitors were used to elucidate the role of downstream mediators of cell death signaling pathways. Adult mice were administered intravitreal injections of 50 micrograms of chloroquine. Fundus photography, optical coherence tomography, and electroretinography were used to evaluate drug-induced retinal changes after one week. Quantitative RT-PCR was used to evaluate changes in gene expression in mice retinal-pigmented epithelium and neurosensory retina.

Results: ARPE-19 and 661W cells showed cell death within 24 hours of treatment. LAMP-1 staining and inhibition of cathepsins B, D, and L activity in ARPE-19 cells significantly decreased cell death, indicating that lysosomal destabilization and cathepsin release occur prior to regulated cell death. Treatment of RPE cells with caspase inhibitors and Nec-1 showed primarily caspase-dependent cell death in RPE cells whereas treatment of photoreceptor cells with caspase inhibitors and Nec-1

showed significant rescue of cell death. Chloroquine-injected animals demonstrated functional visual loss consistent with marked abnormality and disruption of the photoreceptor layer. Gene expression changes showed a significant up regulation of markers specific to caspase mediated cell death in the RPE and necrosis and oxidative stress markers in the neurosensory retina.

Conclusions: ARPE-19 cells underwent both apoptotic and pyroptotic cell death while 661W cells showed activation of apoptosis and necroptosis. Chloroquine-injected mice demonstrated characteristic phenotypes also observed in human patients with chloroquine toxicity and showed activation of various cell death mediators. By elucidating the mechanism of chloroquine toxicity, we believe our findings can shed light on potential therapeutic agents.

Introduction

Chloroquine (CQ) is used for the treatment of malaria and management of rheumatoid arthritis and systemic lupus erythematosus. CQ as well as hydroxychloroquine (HCQ) can induce a dose-dependent maculopathy, leading to irreversible loss of vision. Although the complete mechanism of this drug toxicity is unclear, the disruption of retinal pigmented epithelium (RPE) lysosomal function appears to be common.^{1, 2, 3, 4} It has been proposed that CQ disrupts the lysosomal activity of the RPE, subsequently disrupting cell autophagy and leading to eventual cell death.⁵ CQ retinopathy is associated with RPE degeneration and thinning of the neuroretina with pigmentary changes of the macula, progressing to a pathognomonic “bull’s eye” maculopathy that is characterized by paracentral visual field loss and parafoveal atrophy of the RPE.^{6, 7} The prevalence of HCQ-induced maculopathy has been reported to be 5%–20%, while the prevalence of CQ-induced maculopathy can range from 1%–20% depending on duration of treatment.^{8, 9, 10} Due to the potential associated maculopathy, the American Academy of Ophthalmology recommends baseline exams and yearly screening with multimodal imaging after five years of CQ use.¹⁰

Several studies in humans have shown that while the inner neural retina is preserved, the RPE and the photoreceptor (PR) outer segments are the primary targets of CQ.^{11, 12} To date, it is uncertain whether CQ acts exclusively on RPE cells with PR dying secondarily or it affects the PR outer segments first, causing thinning of the outer nuclear layer (ONL) followed by RPE cell death. Previous studies have suggested that CQ first accumulates in RPE cells due to its affinity to bind to melanin pigment.^{13, 14, 15} Other studies that employ fundus and optical coherence tomography (OCT) imaging in humans, however, have shown progressive thinning of the PR layer, with changes in the RPE layer occurring later only at the onset of bull’s eye maculopathy.^{12, 16}

CQ has also been shown to preferentially accumulate in the pigmented ocular tissues in rabbit and rat models.^{17, 18} Several animal models with acute and chronic exposure to CQ have been studied and have shown morphological changes in the PR and RPE layers.^{1, 10, 16, 19–22} However, to date there are no reproducible mouse models of CQ retinopathy and no report to date has classified the cell death pathways that are involved in CQ-induced RPE and PR death in vitro.

Several studies have shown that CQ, due to its basic nature, diffuses into lysosomes, gets protonated, and then sequesters within lysosomes.^{23, 24} This protonation causes lysosomal pH to rise, thereby inhibiting lysosomal proteases.²⁵ Due to its affinity to melanin pigment, CQ accumulates in the lysosomes of RPE cells.²⁶ Since RPE cells are critical in the maintenance of PRs, functionally defective

RPE cells can lead to PR damage. It has also been observed that accumulation of CQ in the lysosomes causes disruption of cell autophagy and subsequent cell death.

Lysosomal membrane permeabilization (LMP) causes the release of hydrolases called cathepsins from the lumen into the cytosol. These cathepsins activate proteolytic hydrolases such as caspases. Caspase activation leads to caspase-dependent cell death pathways including pyroptosis and apoptosis. Additionally, LMP leads to the activation of caspase independent necrosis.²⁷

Cell death can occur via three major pathways: pyroptosis, apoptosis, and necrosis.²⁸ Cellular stress signals can induce apoptosis, which can lead to DNA damage. Caspases can be classified as initiator caspases (caspase-2, caspase-8, and caspase-9) or as executioner caspases (caspase-3, caspase-6, and caspase-7).²⁹ Apoptosis is initiated by an assembly of adaptor molecules and subsequent cleavage of initiator caspases, which activate downstream executioner caspases -3 and -7. The executioner caspases cause aberrant changes in the actin cytoskeleton and membrane blebbing, a defining feature of apoptosis. Apoptosis can be classified as intrinsic or extrinsic. The intrinsic pathway is triggered in response to cellular stress signals that lead to the release of Bcl2 proteins. Bcl2 proteins trigger the release of cytochrome c into the cytoplasm, leading to the recruitment and activation of caspase-9.^{30,31} The extrinsic pathway is mediated by death receptors such as TNFR1, Fas, and TRAIL, which recruit adaptor proteins and subsequently activate caspase-8.³¹ Caspase-3 is cleaved and activated downstream in both pathways.^{32, 33}

Pyroptosis is induced by inflammatory signals, which lead to the recruitment and activation of caspase-1 by assembly of Nod like receptors (NLRs).^{28, 29, 34} Caspase-1 activation leads to the secretion of two primary inflammatory cytokines, IL-1 β and IL-18.³⁵⁻³⁸

A form of programmed necrosis, also referred to as RIP-kinase dependent necrosis or necroptosis, also occurs due to excessive cellular damage.²⁸ In necroptosis, there is loss of cell membrane integrity, leading to the release of cellular contents. Activation of necrosis involves the recruitment of receptor-interacting serine/threonine protein kinase 1 (RIP1) and RIP3 kinase.³⁹⁻⁴¹ Necroptosis is caspase-independent and lacks the features of apoptosis and pyroptosis.⁴²

In our study, we aim to investigate the mechanisms of chloroquine-induced cell death in vitro in adult retinal pigmented epithelial (ARPE-19) cells and murine 661W photoreceptor-derived cells as well as develop a reproducible preliminary mouse model of chloroquine-induced retinal toxicity. We provide evidence that in ARPE-19 cells, CQ resulted in caspase-dependent apoptosis and pyroptosis, whereas it triggered necrosis and apoptosis in the cone PR cell line 661W. We then developed and characterized a mouse model of CQ-induced acute retinal toxicity with characteristic phenotypes also observed in humans. We also provided evidence of similar underlying cell death mechanisms mediating acute CQ toxicity in the mice RPE and PRs.

Methods

Cell culture of human adult retinal pigmented epithelial (ARPE-19) and 661W cells

Human ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were cultured as described previously.⁴³ After growing to confluence, the cells were maintained in 1% fetal bovine

serum media until used for experiments. For CQ treatment experiments, cells were left in media devoid of serum and starved overnight in DMEM/F12 media with 2 mM L-glutamine and 100 U/mL penicillin. Murine 661W PR-derived cells were plated at 25,000 cells per well in 48-well plates and cultured in regular Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) for 24 hours before the experiments were performed in serum free medium.

Chloroquine treatment

CQ diphosphate (Sigma-Aldrich Corp.) stock solution (50mM) was prepared in sterile distilled water according to the manufacturer's recommendation. Cells were treated with different doses of CQ (10, 50, 100, 150, 250, and 500 μ M). The controls received serum free media containing the same amount of vehicle without CQ.

Assessment of cytotoxicity

To assess CQ-induced toxicity, the conditioned media from ARPE-19 and 155 661W cone PR cells was collected after 24 hours of treatment, and the percentage of cell death was quantified by measuring the LDH release in the conditioned media using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Both cell types were treated with increasing concentrations of CQ. As a positive control, the same number of cells maintained in parallel was lysed by two freeze-thaw cycles; the conditioned media were collected to measure the maximum LDH release. Percentage LDH release was calculated as $100\% \times (\text{experimental LDH} - \text{spontaneous LDH}) / (\text{maximal LDH} - \text{spontaneous LDH})$. There was a significant release of LDH observed at 500 μ M for ARPE-19 cells and 100 μ M for 661W cells; therefore, these concentrations were used to perform all our experiments described below.

Immunofluorescence

To assess the effect of CQ on lysosomal integrity, human ARPE-19 cells seeded on laminin-coated coverslips were treated with CQ or control medium. At 3 hours and 6 hours post treatment, cells were washed with PBS and fixed with 4% paraformaldehyde for 5 minutes, and immunofluorescence staining was performed as previously described in our established protocol.⁴³ Images were obtained using a Zeiss Axioskop 2 MOT Plus microscope (Carl Zeiss Inc.) and analyzed using Zen 2.6 software (blue edition). All the data are represented from images analyzed from at least three independent experiments.

Inhibition of RIP1 kinase, caspases, and cathepsins

Cells maintained in 48-well plates were treated with CQ alone or in combination with inhibitors as follows: Pepstatin A, Cathepsin D Inhibitor, specific cathepsin B inhibitor CA-074-Me, and/or cathepsin L inhibitor Z-FY (t-Bu)-FMK, RIP1 kinase inhibitor, necrostatin-1 (30 μ M; Sigma-Aldrich Corp.); pan-caspase inhibitor, Z-VAD-FMK, caspase-1 inhibitor Z-YVAD-FMK, caspase-3 inhibitor Z-DEVD-FMK, caspase-8 inhibitor Z-IETD-FMK, caspase-9 inhibitor Z-LEHD-FMK (50 μ M; all from BioVision, Mountain View, CA, USA); (50 μ M; all from EMD Millipore, Billerica, MA, USA).

Animals

Four-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME) ordered and used in this study were approved by our institutional animal care and use committee and all procedures involving the use of animals were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Chloroquine injection

4-week-old mice were anesthetized with an intraperitoneal injection of ketamine/ xylazine (120 mg/kg ketamine and 20 mg/kg xylazine) using a 0.5", 27G needle. Animals under anesthesia were kept warm using a heating blanket. Mice pupils were dilated using a drop of 1% tropicamide applied on the corneal surface. Each mouse received a single injection of the experimental CQ in the right eye and vehicle control agent (sterile saline) in the left eye. CQ was solubilized at a concentration of 50 mg/ml into sterile saline. Experimental mice received 50 micrograms of CQ in 1 microliter of agent. A 30 G needle through the conjunctiva and sclera was used to create a small injection site. Then, using a 32 G Hamilton needle, 1 microliter of agent was injected directly into the vitreous. The surface of the eye was lubricated with Genteal ointment. Mice were monitored post-injection days 1 and 2, and at each experimental time point (14 and 28 days after injection). Following injection, the eyes were dressed with a triple antibiotic (Neo/poly/bac) ointment. To reduce potential post-operative pain, mice received a subcutaneous injection of Meloxicam at the end of the procedure.

Spectral domain optical coherence tomography (SD-OCT) and fundus photography

Ultra-high-resolution SD-OCT images of the retina were acquired using the Bioptigen SD-OCT (Bioptigen, Morrisville, NC). A Micron III (Phoenix Research Laboratories, Pleasanton, CA) system was used for taking fundus images according to manufacturers' instructions. Proper alignment was ensured using the fundus camera in the optical head of the instrument.

Electroretinography (ERG)

Functional assessment of animals was performed with electroretinogram (ERG) recordings of both left and right eyes of each mouse. CQ-treated and control mice were adapted to the dark overnight. Following overnight dark adaptation, the animals were prepared for ERG recordings under dim red light. While under anesthesia with a mixture of Ketamine (120 mg/kg i.p.) and Xylazine (20 mg/kg i.p.), the animal body temperature was maintained at 38°C using a warm heating blanket, and their pupils were dilated using a 0.5% tropicamide ophthalmic solution applied on the corneal surface. A drop of genteal (corneal lubricant) was applied to the cornea of the untreated eye to prevent dehydration, and a drop 0.9% sterile saline was applied to the treated eye to prevent dehydration. A 25-gauge platinum needle, inserted subcutaneously in the forehead, served as a reference electrode, while a needle inserted subcutaneously near the tail served as a ground. A series of flash intensities were produced by a Ganzfeld color dome controlled by the Diagnosys Espion3 to test both scotopic and photopic responses.

Histological evaluation

For histological evaluation, whole eyes were enucleated, fixed in 10% formalin overnight, washed with phosphate buffered saline (PBS), and prepared for paraffin embedding. Haematoxylin and eosin-stained slides were evaluated and the thickness of the retinal layers determined. For each section, measurements were taken from and close to the optic nerve. Five images were taken per section and averaged.

Transmission electron microscopy

Mouse eyes were enucleated and immersion fixed with half strength Karnovsky's fixative (2% formaldehyde + 2.5% glutaraldehyde, in 0.1 M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Hatfield, Pennsylvania) at room temperature. An eyecup was created from each eye by dissecting away the anterior from the posterior ends. Eyecup samples were then placed back into the half strength Karnovsky's fixative for a minimum of 24 hours under refrigeration. After fixation, samples were rinsed with 0.1M sodium cacodylate buffer, post-fixed with 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 1.5 hours, en bloc stained with 2% aqueous uranyl acetate for 30 minutes, then dehydrated with graded ethyl alcohol solutions, transitioned with propylene oxide and resin infiltrated in tEPON-812 epoxy resin (Tousimis, Rockville, Maryland) utilizing an automated EMS Lynx 2 EM tissue processor (Electron Microscopy Sciences, Hatfield, Pennsylvania.) Processed tissues were oriented in tEPON-812 epoxy resin and polymerized in silicone molds using an oven set for 60°C. Semi-thin cross sections for light microscopy were cut at 1-micron and stained with 1% toluidine blue in 1% sodium tetraborate aqueous solution for assessment and screening regions of the tissue block face for thin sectioning. Ultrathin sections (70-80 nm) were cut from the epoxy block using a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL) and a diamond knife, collected onto 2×1mm single slot formvar/carbon coated grids. The thin sections were imaged using a FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, Oregon) at 80 kV interfaced with an AMT XR41 digital CCD camera (Advanced Microscopy Techniques, Woburn, Massachusetts) for digital TIFF file image acquisition. TEM imaging of all layers of the retina was assessed and images were captured at representative regions.

Visualization of RPE morphology

For visualization of the RPE cytoskeleton, immunofluorescence was performed on the CQ- and control-treated eyes. The eyes were fixed in 4% paraformaldehyde overnight and washed with 1X PBS. Eyecups with RPE-choroid were dissected out for each of the eyes. The eyecups were blocked in a blocking buffer containing 5% goat serum in PBS and 0.2% Triton X-100 for an hour at room temperature (RT). RPE-choroid flat mounts were incubated for 4 hours at RT with an Alexa 488 phalloidin antibody at a 1:100 concentration in 5% goat serum in PBS buffer. The eyecups were washed with PBS and flattened to mount on a slide. After mounting, images were taken with a Zeiss Axioscope (Carl Zeiss microscopy, White Plains, New York).

Immunofluorescence for caspase detection

For immunofluorescence, paraffin embedded sections obtained above for histology were used for cleaved caspase-3 staining. Sections were processed through a series of washes that included 100%

Xylene, ethanol, and then distilled water. Deparaffinization was followed by antigen retrieval using sodium citrate buffer. Slides were incubated in the boiling sodium citrate buffer in a pan for about 20 minutes followed by 20 minutes of cooling at RT. Sections were then permeabilized using 0.2% Triton X-100 in PBS for 20 minutes and blocked in 5% normal goat serum for an hour. Slides were incubated with the primary antibody rabbit anti-cleaved caspase-3; (1:100, Cell signaling; Danvers, MA, USA) at 4°C overnight. Three washes with PBS was followed by 2 hours of incubation in a secondary antibody goat anti-rabbit Alexa Fluor 594 (1:300; Life Technologies, Carlsbad, CA). Slides were washed and mounted using a Prolong Gold antifade reagent 4', 6-diamidino-2-phenylindole (DAPI; Life Technologies). Images were obtained using Zeiss Axioscope (Carl Zeiss microscopy, White Plains, New York).

RNA extraction from RPE cells and quantitative PCR (qPCR)

RNA isolation was performed from posterior eyecups with RPE-choroid of CQ-treated and control-treated eyes using the simultaneous RPE cell isolation and RNA stabilization (SRIRS).⁴⁴ The posterior eyecup after sterile dissection was transferred into a 1.5ml tube with 200ul of RNA stabilization reagent, RNAlprotect according to the protocol. Displaced RPE cells were collected, and total RNA was extracted and reverse transcribed as described.⁴⁵ Real-time PCR reactions were performed using the Syber Green Master Mix (SYBR Green; Roche, Basel, Switzerland) and the Lightcycler 480 (Roche Applied Science, Indianapolis, IN). Fold changes in the various cell death markers were quantified by normalizing the data to the housekeeping genes. The list of the primers used for the genes are listed in Table 1.

Results

Chloroquine induces cell death and lysosomal destabilization in ARPE-19 cells

ARPE-19 cells were treated with escalating doses of CQ, which induced cell death (Figure 1A). ARPE-19 cells after 500μM treatment were stained for LAMP1 to assess lysosomal integrity and to test if CQ treatment induces RPE cell death through lysosomal destabilization. The initial control showed LAMP1 highly concentrated within the lysosomes in RPE cells. However, 3 hours after treatment, there was a significant reduction in the number of cells and increased diffusion of LAMP-1 outside of the lysosomes. At six hours, the cell count was further reduced (signifying cell death), and cytoplasmic staining of LAMP-1 was observed (Figure 1B).

Inhibition of cathepsins, pyroptosis, and apoptosis reduces chloroquine cell death in ARPE-19 cells

To elucidate the primary cell death pathways, ARPE-19 cells in 500μM CQ were treated with pyroptosis, apoptosis, necroptosis, and cathepsin inhibitors. Necrostatin-1, an inhibitor of RIP1 kinase, was used to test the role of necroptosis in CQ-induced cell death.

Following lysosomal destabilization, cathepsins are released into the cytosol leading to activation of caspases. Our findings support this claim. While control cells showed 31.79% (SEM: ± 3.80) LDH release, cells treated with Cathepsin B inhibitors showed a significant reduction in LDH release to 10.61% (SEM: ± 1.91). Combinatorial treatment with Cathepsin B and D inhibitors also showed a

further reduction in LDH release to 8.47% (SEM: $\pm$ 1.24), whereas cells treated with Cathepsin B and L inhibitors also showed significant reduction 3.83% (SEM: $\pm$ 1.05). Cathepsin D or L inhibitors showed no significant reduction in LDH release, showing 23.50% (SEM: $\pm$ 3.68) and 25.39% (SEM: $\pm$ 4.38) LDH release, respectively (Figure 1C).

To assess the relative contribution of various cell death mechanisms to CQ-induced cell death, we treated cells with inhibitors specific for caspase-1 (Z-YVAD-FMK) to investigate the role of pyroptosis, caspase-3 (Z-DEVD-FMK) to investigate the role of apoptosis, and Nec-1 to assess necroptosis. Control cells treated with vehicle and the cells treated with both CQ and Nec-1 showed 31.87% (SEM: $\pm$ 1.76) and 29.56% (SEM: $\pm$ 2.40), respectively. Treatment with caspase-1 inhibitor or caspase-3 inhibitor reduced the LDH release to 20.31% (SEM: $\pm$ 2.3), and 14.28% (SEM: $\pm$ 1.98), respectively. Combinatorial treatment of Nec-1 with either Z-YVAD-FMK or Z-DEVD-FMK did not further reduce LDH release (Figure 1D).

We also used the caspase-8-specific inhibitor (Z-IETD-FMK) and caspase-9-specific inhibitor (Z-LEHD-FMK) to specifically investigate the role of intrinsic and extrinsic apoptosis pathways. Inhibition of caspase-8 significantly reduced LDH release to 12.51% (SEM: $\pm$ 3.44), but inhibition of caspase-9 did not exhibit any significant reduction in cell death (Figure 1E).

Inhibition of necroptosis and apoptosis reduces chloroquine cell death in 661W cells

661W cells in CQ treated with Z-YVAD-FMK (caspase-1 inhibitor) showed no significant reduction in LDH release. However, treatment with Z-DEVD-FMK (caspase-3 inhibitor) reduced cell death from 46% (SEM: $\pm$ 0.85) to 35.21% (SEM: $\pm$ 4.54), indicating that caspase-mediated cell death in PRs is dominated by apoptosis. Hence, the treatment with Z-VAD-FMK (pan-caspase inhibitor) reduced cell death by virtually the same amount as the caspase-3 inhibitor (Figure 1G).

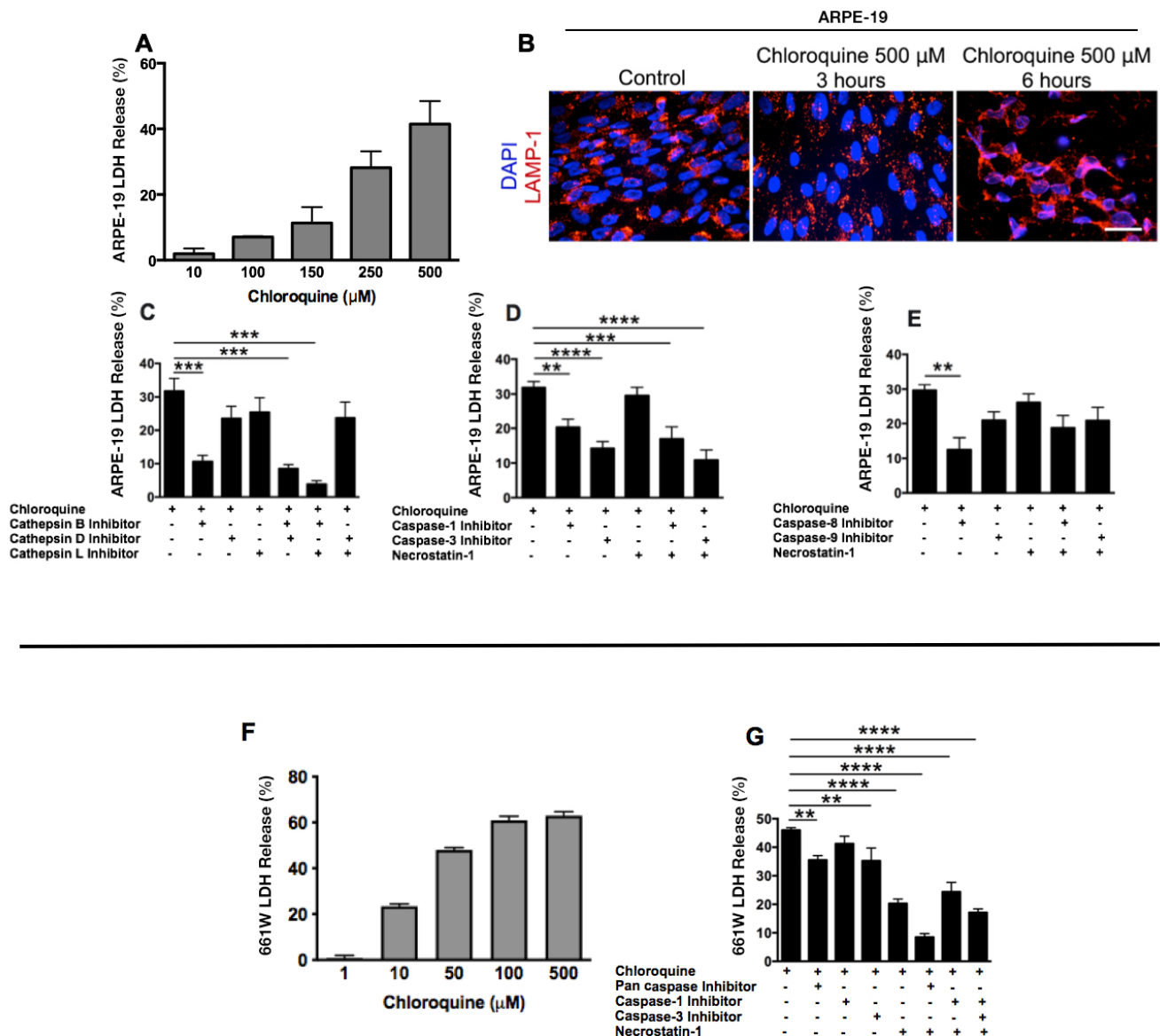
Treatments with Nec-1 showed the largest reductions in cell death, indicating that necroptosis plays a major role in PR cell death. Additional treatment with Z-YVAD-FMK did not lead to any further reduction in LDH release, but combinatorial treatment with the Z-VAD-FMK (pan-caspase inhibitor) led to the greatest reduction in LDH release to 8.48% (SEM: $\pm$ 1.24) (Figure 1G).

Validation of preliminary mouse model

After elucidating the mechanisms of chloroquine-induced acute cell death in ARPE-19 cells and 661W cells in vitro, we then began validating an in vivo mouse model. From our preliminary experiments in C57BL/6J mice, very little to mild toxicity was observed after intra-peritoneal injections or subretinal injections of chloroquine at various time points (7, 14, and 28 days) and even at higher doses. However, we observed appreciable retinal toxicity with intravitreal injection when compared to other routes of administration. For intravitreal injections, we observed similar anatomic, functional, and histopathologic changes in mice administered 0.5 micrograms, 5 micrograms, and 50 micrograms. In addition, we investigated different time points and found similar anatomic, functional, and histopathologic changes at 7, 14, and 28 days after intravitreal injections. Therefore, for all our studies reported herein, phenotypical changes 1-week after 50 micrograms intravitreal chloroquine injections were reported.

Figure 1. ARPE-19 show lysosomal destabilization with induction of apoptosis and pyroptosis, and 661W cells show activation of apoptosis and necroptosis.

(A) LDH analysis show that CQ induces cytotoxicity in ARPE-19 cells. Increasing doses of CQ treatment led to an increase in LDH release. **(B)** LAMP-1 (red) was concentrated in the ARPE-19's lysosomes in the control cells (left). After treatment at 500 μ M, LAMP-1 loss was time-dependent, showing increased LAMP-1 diffusion at 3 hours and even more at 6 hours. **(C)** LDH release of treatment of CQ treated ARPE-19 cells along with Cathepsin B, D, and L inhibitors showed significant reduced cell death with cathepsin B inhibitor alone and in combination with D and L inhibitors. **(D)** Treatment of ARPE-19 cells with caspase-1 (YVAD) and caspase-3 (DEVD) inhibitors caused a significant reduction alone and in combination with Necrostatin (Nec-1) in LDH release but no significant effect on treatment with Nec-1 alone was observed. **(E)** Inhibition of caspase-8, but not caspase-9 significantly reduced LDH release. **(F)** Increasing doses of CQ treatment led to an increase in LDH release in 661W PR cells. **(G)** LDH release of treatment of CQ treated 661W cells along with pan caspase inhibitor (z-VAD), and caspase-3 (DEVD) inhibitors caused a significant reduction alone and in combination with Nec-1 in LDH release. No significant effect on treatment with caspase-1 (Y711 VAD) alone was observed. Scale bar: 25 μ m. Error bars: $\pm$ SEM. ** P < 0.005, *** P < 0.0005, **** P < 0.0001.



Morphological changes in retinal tissue after chloroquine administration

To evaluate the ocular effects of 50 micrograms of intravitreal CQ, fundus photographs 7 days post intravitreal injections were collected and compared to the saline/control-injected animals (Figure 2A-H). Retinal degeneration and significant pigmentary changes of the RPE layer were visible at day 7. SD-OCT evaluation showed significant damage to the ONL at day 7 (Figure 2B, F), and fundus photographs showed disruption of the RPE layer in CQ-treated animals (Figure 2A,E). The OCT images showed several sub-retinal deposits, which were confirmed by the hyperreflective dots observed in the autofluorescence images (Figure 2C,G). The pinpoint fluorescence observed by autofluorescence was only observed in CQ-treated mice and can be postulated to be sick RPE or RPE debris. Retinal vasculature and optic nerve tissue appeared to remain normal at 7 days.

Results from fundus, OCT, and autofluorescence were confirmed by histological and electron micrographic (EM) examinations of ocular specimens. RPE swelling and depigmentation were observed in the CQ-injected animals (Figure 3A). Significant disorganization of the PRs and thinning of the entire retina, especially the outer segments of the ONL and outer plexiform layer (OPL), were observed. Cystic spaces at the inner segment layer and the inner segment-outer segment junction were also observed only in the CQ-treated mice (Figure 3A). Quantification of ONL, inner and outer segment layer thickness, and rows of ONL nuclei demonstrated significant PR degeneration, leading to the thinning of the retina (Figure 3B).

To better characterize changes of the RPE monolayer, we used immunostaining for phalloidin on flat-mounted posterior eyecups. As seen in the saline-injected samples, RPE cells presented with their normal cuboidal shape and even distribution; however, CQ-injected samples showed large areas of RPE atrophy and significantly enlarged and disorganized RPE cells compared to controls (Figure 3C).

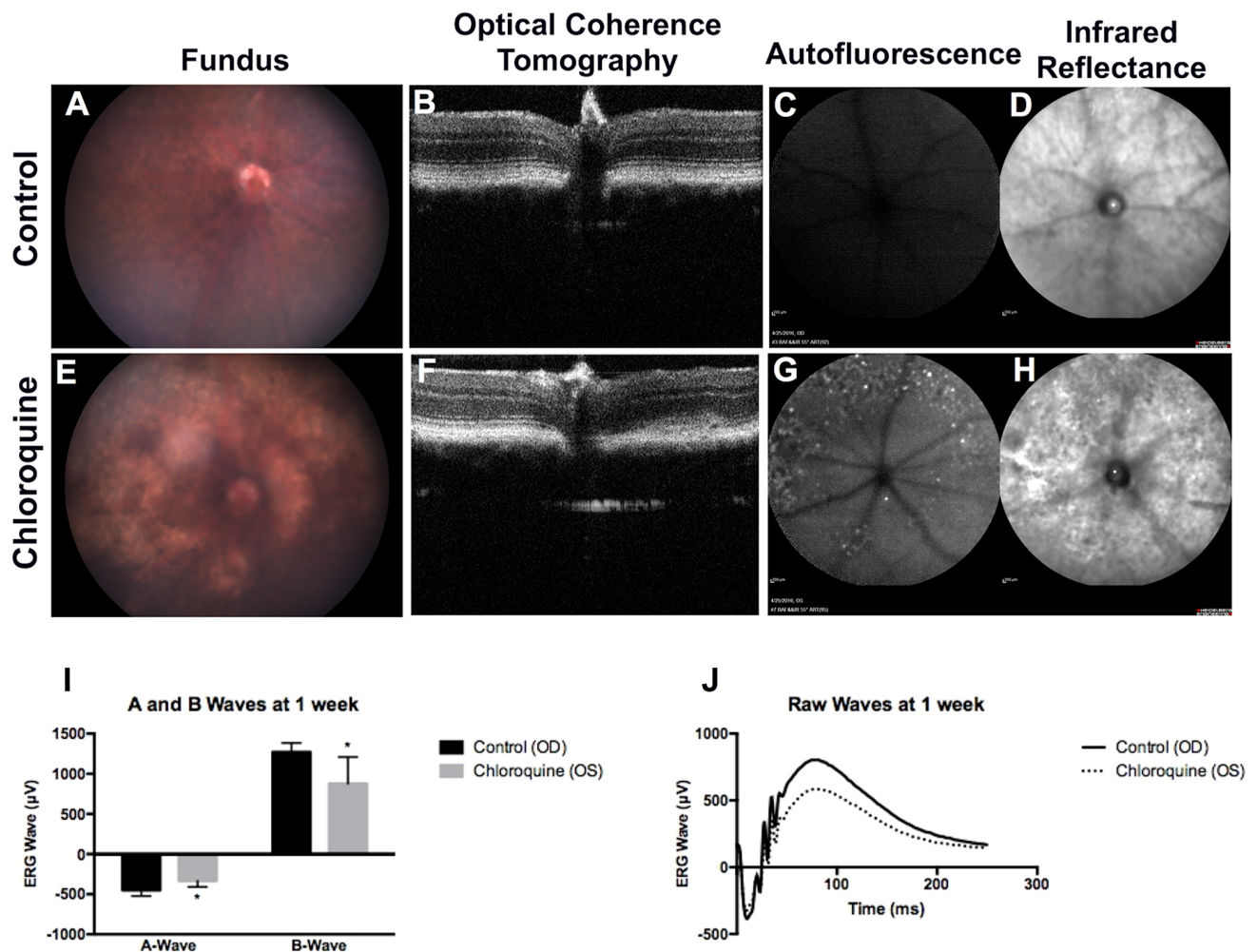
Transmission electron micrograph images of chloroquine-treated eyes (Figure 4B-D) showed morphological changes compared to control eyes (Figure 4A). Loss of RPE nuclei with pigmentary changes and outer segment disruption were observed in chloroquine-treated animals and are depicted by arrows (Figure 4B). Missing portions of the RPE layer and debris accumulation (Figure 4C) as well as empty spaces due to the disruption of the outer nuclear layer were observed (Figure 4C-D).

Characterization of functional effects following chloroquine treatment

Changes in retinal function were examined by ERG 1-week before the CQ injection at baseline and then 1-week post injection. Consistent with our findings from our anatomical examination, a significant reduction in the ERG response was observed both in “a” and “b” waves in CQ-treated animals compared to saline-injected animals (Figure 2I-J). A smaller amplitude was observed in the saline-injected compared to the un-injected controls. The reduction in amplitude can be attributed to the injection, but this reduction was not significant.

Figure 2. Fundus photography and optical coherence tomography show chloroquine-induced damage of the retina.

Color fundus, OCT, autofluorescence, and infrared reflectance photographs of C57BL/6J mice treated with CQ reveal **(E)** subretinal drusen-like deposits, loss of RPE with pigmentary changes, **(F)** PR layer and outer retinal disruption, **(G)** pinpoint hyperautofluorescence, and **(H)** extensive pigmentary changes on IR when compared to control **(A-D)**. Analysis of visual function of C57BL/6J mice treated with CQ showed a significant reduction in the ERG responses at 1-week. **(I)** Both 'a' wave and 'b' wave were significantly reduced compared to the control. **(J)** A representative wave comparing control and CQ-injected mice. Error bars: $\pm$ SEM. * $P < 0.05$.



Induction of cell death pathway genes by chloroquine in retinal pigment epithelium and the retina

Cells of the ONL of mice were stained via immunofluorescence for caspase-3. Caspase-3 was detected within the ONL in the CQ-treated mice but not detected in the saline-injected control mice (Figure 3D). The acute effects of CQ were further evaluated by quantifying changes in expression of cell death, oxidative stress, and cathepsin-related genes using qPCR on isolated RPE and neuroretina (free of RPE) samples one-week post CQ injection. Intravitreal treatment with CQ was associated with a dramatic loss of RPE65 expression (Figure 5A). Induction of cathepsins B, D, and L expression was observed (Figure 5B). Increased expression of pyroptosis markers such as Nlrp2, Nlrp3, and ASC—the inflammasome assembly—was observed as well as an increase in the ratio of Bax / Bcl2 expression (pro-apoptotic) (Figure 5C). However, there was no significant effect on the oxidative stress markers Gpx1, Gpx4 and Sod2 in the RPE cells (Figure 5D). There was also no significant

effect on the necroptosis marker RIP3 (Figure 5C). In the outer retina, there was no significant change in the expression of rhodopsin and opsin genes, but there was significant down-regulation of Peripherin-2, a gene found in rods and cone cells of the retina. Also, a significant up-regulation of Gpx4, RIP3, and Bax / Bcl2 genes was observed in the neuroretina (Figure 5E-H).

Statistics

Data are presented as mean $\pm$ SEM of at least three independent experiments unless otherwise indicated. Statistical significance was evaluated using a one-way ANOVA followed by post hoc Tukey-Kramer multiple comparison tests, using the Prism 6.0 software package (GraphPad Software, Inc., La Jolla, CA, USA). Adjusted P values < 0.05 were considered statistically significant.

Figure 3. Chloroquine alters retinal layer thicknesses and activates caspase-3.

(A) Hematoxylin and eosin staining of paraffin-embedded retinal cross sections showed altered outer retina thickness following 1-week post-CQ injection. Immunohistochemistry showed RPE swelling and depigmentation, disruption of PRs, and loose organization of the outer nuclear and inner nuclear layers with cystic spaces in CQ injected eyes compared to saline injected eyes. **(B)** The thickness of different layers of the retina were measured and quantified on histological sections. CQ-treated mice showed statistically significant degeneration of the outer segments with thickness of $15.65 \mu\text{m}$ (SEM: ± 3.23), while saline injected mice showed $30.87 \mu\text{m}$ (SEM: ± 5.17). CQ treated mice also showed significant degeneration of the ONL $76.63 \mu\text{m}$ (SEM: ± 6.13) while control showed $94.9 \mu\text{m}$ (SEM: ± 3.70). Degeneration of the OPL $7.45 \mu\text{m}$ (SEM: ± 1.81) was also seen compared to controls $25.44 \mu\text{m}$ (SEM: ± 2.29). **(C)** Immunostaining for phalloidin showed that saline injected eye (left) had normal RPE morphology and distribution. CQ treated animals showed highly disorganized and enlarged RPE cells and spots of RPE atrophy. **(D)** Immunofluorescence staining of paraffin embedded sections with cleaved caspase-3. Active caspase-3 positive cells were detected in the ONL, OPL, and RPE cell layers (middle row) in CQ-injected mice but not in saline injected eyes (top row). Scale bar: $100 \mu\text{m}$ (3A, and 3D); $50 \mu\text{m}$ (3C). Error bars: $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

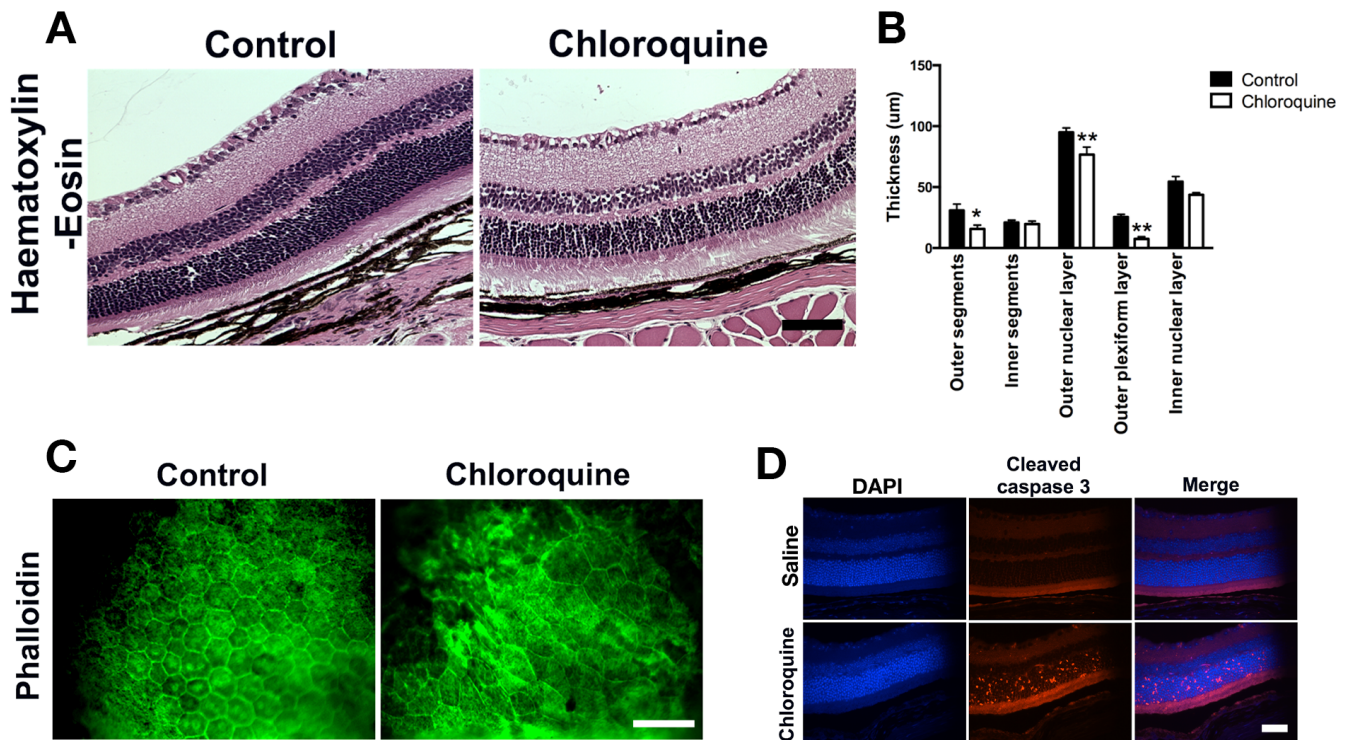


Figure 4. Transmission electron micrographs showed chloroquine-induced damage of the outer nuclear layer.

Transmission EM images of chloroquine-treated eyes (**B-D**) showed morphological changes compared to control eyes (**A**). Loss of RPE nuclei with pigmentary changes and outer segment disruption were observed in chloroquine-treated animals (**B**). Missing portions of the RPE layer and debris accumulation as well as empty spaces due to outer nuclear layer disruption were observed (**C-D**).

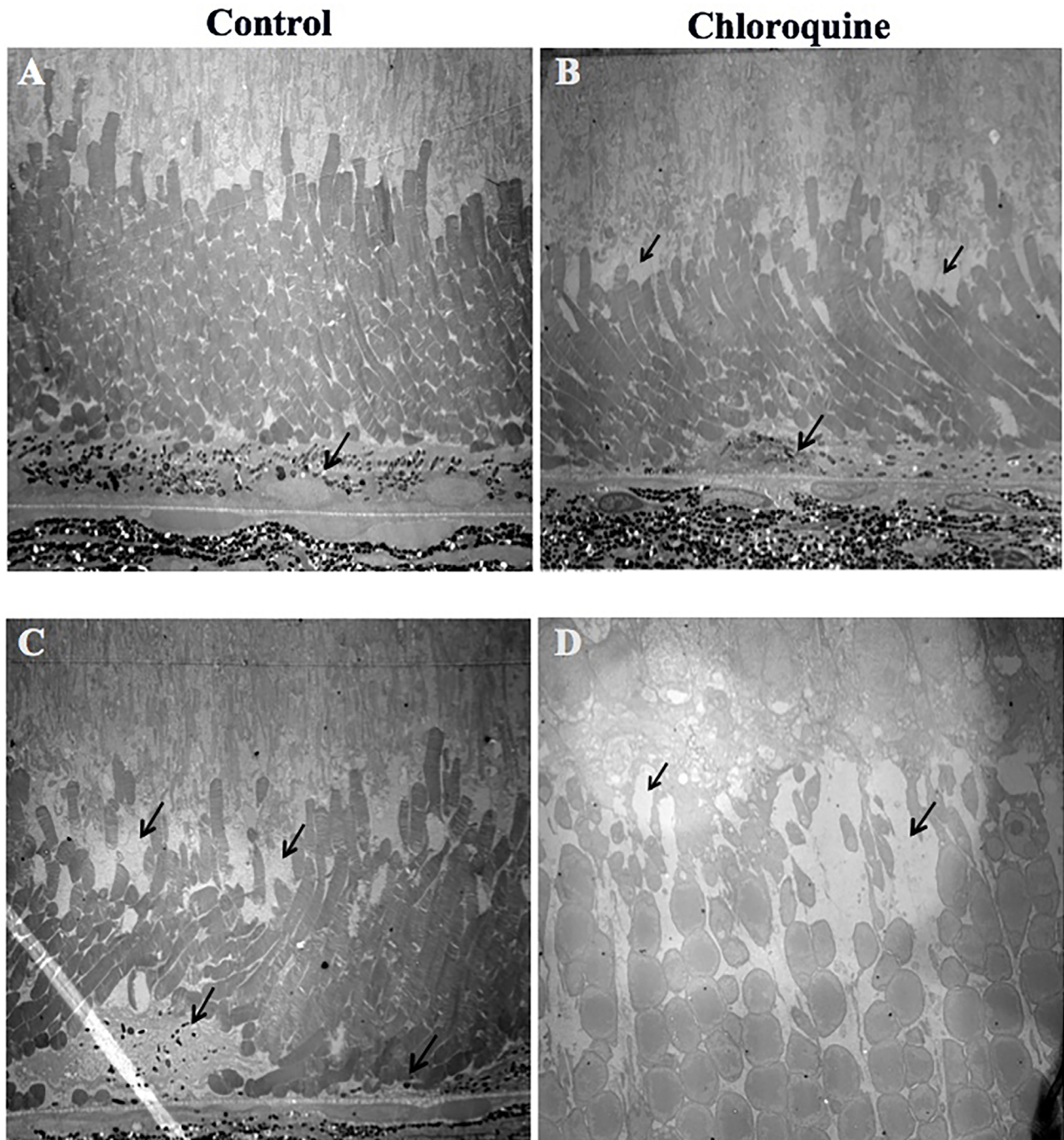
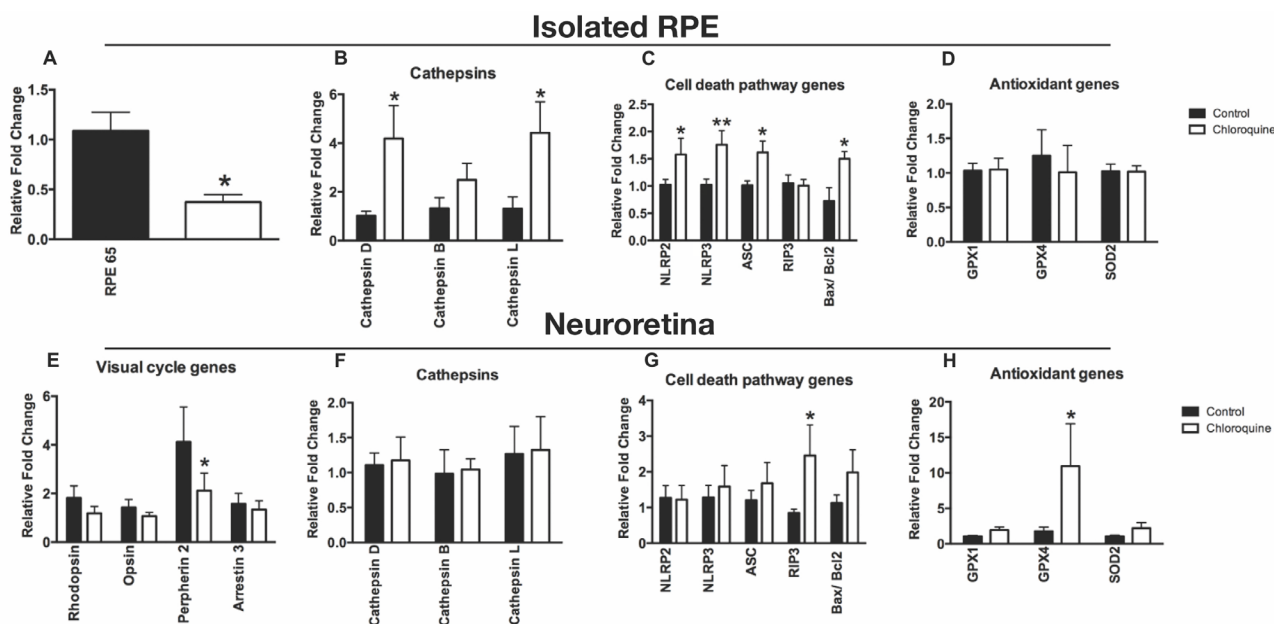


Figure 5. Isolated RPE and neurosensory retina showed gene expression consistent with cell death activation.

Analysis via q-PCR indicates that CQ toxicity induces cell death and up regulation of cell death markers, Cathepsins, and antioxidant genes. After treatment with CQ, RPE cell death **(A)** and significant up regulation of Cathepsins D and L was observed **(B)**. While up regulation of cell death pathway genes NLRP2, NLRP3, and ASC were observed **(C)**, up regulation of Antioxidant genes was not observed **(D)**. In the neuroretina, no significant increase of Rhodopsin and Opsin was observed but a significant decrease in Peripherin-2 was observed **(E)**. A significant transcriptional increase in the RIP3 cell death pathway gene **(F)** and in the GPX4 Antioxidant gene **(H)** was observed. **(G)** No significant up regulation of Cathepsins was observed in the neuroretina. Error bars: $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.



Discussion

CQ toxicity has been described in the scientific literature for over 50 years.⁴⁴ We confirmed the effects of CQ on lysosomal destabilization in vitro and provide evidence that the subsequent release of cathepsins plays a major role in initiating caspase-dependent apoptosis and pyroptosis in ARPE-19 cells. Interestingly, in 661W cells, CQ induced apoptosis, necroptosis, and oxidative stress. To date, there are no reproducible mouse models for CQ retinopathy. After elucidating the in vitro mechanisms of chloroquine-induced cell death, we developed and characterized a mouse model of CQ-induced acute retinal toxicity with characteristic phenotypes also observed in humans. We also provided evidence of similar underlying cell death mechanisms mediating acute CQ toxicity in the mice RPE and PRs.

Consistent with the literature, we observed lysosomal destabilization shortly after ARPE-19 cells were treated with CQ. We have shown that lysosomal permeabilization releases cathepsins, leading to cell death, and specific cell-permeable inhibitors for cathepsins B, L, and D significantly reduce cell death. Lysosomal destabilization and the associated release of cathepsins have been shown to activate the NLRP3 Inflammasome in the RPE.^{42, 43, 46} Inhibition of inflammasome assembly and/or caspase-1 activation can reduce pyroptosis induced cell death. Inhibition of caspase-1 partially reduced CQ-induced ARPE-19 cell death. Pathologic activation of NLRP3 inflammasome and activation of pyroptosis have also been widely studied in a variety of diseases in which lysosomal destabilization

can be induced by various stimuli like AluRNA, cholesterol crystals, and silica crystals.⁵⁰⁻⁵² The NLRP3 inflammasome has been shown to be activated in the RPE of patients with AMD and is hypothesized to play a role in the disease pathogenesis.³⁸ Similarly, from our results we extrapolate that the NLRP3 inflammasome and pyroptosis are major mediators of CQ-induced RPE cell death.

Our previous study that investigated tamoxifen toxicity of ARPE-19 cells in vitro also found that lysosomal destabilization and subsequent cell death occurred after tamoxifen treatment.⁴³ However, the onset of lysosomal destabilization after tamoxifen treatment was seen within 90 minutes, unlike ARPE-19 cells after CQ treatment, which showed destabilization within 3 hours. Both CQ- and tamoxifen-treated ARPE-19 cells showed a statistically significant reduction in LDH release after inhibition of caspase-1 and showed an even greater reduction in LDH release after caspase-3 inhibition. However, unlike tamoxifen-treated RPE cells, inhibition of necroptosis was not cytoprotective in CQ-treated ARPE-19 cells.⁴³

A series of studies indicate that not only apoptotic but also autophagic and necrotic signaling are involved in PR cell death. These studies suggest that targeting these pathways in tandem may be an effective neuroprotective strategy for retinal diseases associated with PR cell loss. Interestingly, inhibition of caspase-3 (with Z-DEVD-FMK) and RIP1 (with Necrostatin-1) alone and in combination in CQ-treated 661W PR cells significantly reduced cell death.

Cysteine proteases from the caspase family play a major role in apoptosis, and their activation can be achieved in several ways. Caspases orchestrate the morphological and biochemical changes that characterize apoptosis. Caspase-8 and -9 mediate the extrinsic and intrinsic pathways of apoptosis, respectively; and both initiator caspases can activate caspase-3. Inhibition of caspase-8 and caspase-9 showed some reduction in cell death whereas inhibition of caspase-3 caused a significant reduction in CQ-induced ARPE-19 cell death.

After elucidating the mechanism of chloroquine-induced cell death in ARPE-19 and 661W cells in vitro, we reassessed the mechanisms of CQ-induced retinal damage using a C57BL/6J mouse model. We examined the effects of different doses and post-injection time points on both mice retinal structure and function. In addition, we examined the effects of different routes of chloroquine administration and found very little to mild toxicity after intra-peritoneal injections or subretinal injections of chloroquine at various time points and at higher doses. However, we observed appreciable retinal toxicity with intravitreal injection when compared to other routes of administration. Although only data for 50 micrograms of intravitreal CQ were shown, similar effects were observed in mice administered intravitreal injections of lower doses of 5 micrograms and 0.5 micrograms at different time points. CQ led to significant loss of retinal function at day 7, local morphological changes in RPE cells and PRs, and thinning of the outer retina associated with degeneration of RPE, rods, and cones, leading to the significant ablation of the outer nuclear and RPE layers.

The results observed by our group are parallel to studies observed in various animal models with rats, mice, and rabbits. These studies have also shown retinal function loss in these animals administered CQ orally, intraperitoneally, intramuscularly, and via osmotic pumps.^{1, 14-18} Further abnormalities in the ONL, demonstrated by complete loss of the OPL as well loss of PR layers along with RPE atrophy and pigment irregularity, observed in our model have been 495 confirmed by other animal models. Although it is difficult to compare our results to previously published reports, due to

differences in the source of CQ, methods of administration, and time points analyzed after injection, trends in changes can still be meaningfully compared.

There is controversy over whether RPE cells are the primary targets for CQ. One study found that CQ targets the ONL first, with RPE changes resulting later.⁴⁷ However, none of the previous animal models have been able to definitively address this question. In our study, we observed acute notable changes both to the RPE and ONL layers at the one-week time point. We observed a significant reduction in both the a- and b-wave amplitudes, implying the presence of defects in the PRs and depolarizing bipolar cells. Vacuoles and cystic spaces were observed in the CQ-injected mice, contributing to the functional changes observed. A study using rat animal models observed morphological changes associated with UV auto-fluorescence without evidence of retinal gliosis 3 months after oral CQ administration.⁴⁸ We observed similar results after our administration of CQ; treated animals showed hyperreflective dots observed by autofluorescence. A pinpoint fluorescence was observed by autofluorescence that can be postulated to be sick RPE or RPE debris.

Although it is known that CQ-induced lysosomal destabilization can trigger RPE cell death, the downstream mechanism is unclear. Gene expression analysis of the RPE layer one-week post CQ treatment showed that RPE-specific marker RPE65 was significantly downregulated, clearly proving that the RPE layer is affected by CQ treatment. We also observed in the RPE the induction of inflammasome assembly genes Nlrp2, Nlrp3, and ASC involved in pyroptosis along with an increase in the ratio of Bax/ Bcl2, a pro-apoptotic marker. In the RPE, we observed no effect on RIP3 gene activation, an important mediator of necrosis, though RIP1 activation was observed in an in vitro model of tamoxifen toxicity.⁴³ On the other hand, gene expression analysis of the PRs showed induction of the antioxidant gene Gpx4 that protects cell from lipid peroxidation. There is evidence in the literature that CQ induces lipid peroxidation in the retina of a rat.⁴⁹ We also observed the induction of Bax/Bcl2 and RIP3 in the PRs. Similarly, the ONL layer of mice injected with CQ showed cleaved caspase-3, unlike the controls.

The results suggest that CQ-induced ARPE-19 cell death is triggered by lysosomal membrane permeabilization, leading to the release of cathepsins that trigger the caspase-mediated cell death pathways, apoptosis and pyroptosis, as well as necroptosis. In 661w photoreceptor cells, cell death mechanisms were investigated using specific inhibitors. While inhibiting caspase-1 had no effect on LDH release (a measure of cell membrane damage), inhibiting caspase-3 significantly reduced cell death, suggesting apoptosis is a key pathway. However, the most substantial reduction in cell death occurred when necroptosis was inhibited, indicating that necroptosis plays a dominant role in photoreceptor cell death in this model. Our results indicate that intravitreal administration of CQ in a mouse model triggers rapid retinal dysfunction, measurable RPE atrophy, and adverse effects on the PRs. We also provided evidence of similar underlying cell death mechanisms mediating acute CQ toxicity in the mice RPE and PRs. Understanding the mechanisms of cell death in CQ retinopathy is important for identifying therapeutic agents, and future studies should further explore these specific cell death pathways as potential candidates for therapies.

Conflicts of interest

The authors declare no potential conflicts of interest.

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Authors' contributions

- **Dhanesh Amarnani:** Data Curation, Formal Analysis, Methodology, Writing (Original Draft Preparation)
- **Brandon Duffy:** Data Curation, Methodology
- **Eric J. Kim:** Data Curation, Methodology, Writing (Original Draft Preparation)
- **Leslie Ramos:** Data Curation, Methodology
- **Leo A. Kim:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Supervision, Writing (Original Draft Preparation), Writing (Review & Editing)

References

1. Mahon GJ, Anderson HR, Gardiner TA, McFarlane S, Archer DB, Stitt AW. Chloroquine causes lysosomal dysfunction in neural retina and RPE: implications for retinopathy. *Curr Eye Res.* 2004; 28:277-84. DOI: [10.1076/ceyr.28.4.277.27835](https://doi.org/10.1076/ceyr.28.4.277.27835)
2. Sundelin SP, Terman A. Different effects of chloroquine and hydroxychloroquine on lysosomal function in cultured retinal pigment epithelial cells. *APMIS.* 2002; 110:481-9. DOI: [10.1034/j.1600-0463.2002.100606.x](https://doi.org/10.1034/j.1600-0463.2002.100606.x)
3. Toimela T, Tähti H, Salminen L. Retinal pigment epithelium cell culture as a model for evaluation of the toxicity of tamoxifen and chloroquine. *Ophthalmic Res.* 1995; 27:150-3. DOI: [10.1159/000267861](https://doi.org/10.1159/000267861)
4. Li X, Fei J, Lei Z, Liu K, Wu J, Meng T, Yu J, Li J. Chloroquine impairs visual transduction via modulation of acid sensing ion channel 1a. *Toxicol Lett.* 2014; 228:200-6. DOI: [10.1016/j.toxlet.2014.05.008](https://doi.org/10.1016/j.toxlet.2014.05.008)
5. Yoon YH, Cho KS, Hwang JJ, Lee S-J, Choi JA, Koh J-Y. Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells. *Invest Ophthalmol Vis Sci.* 2010; 51:6030-7. DOI: [10.1167/iovs.10-5278](https://doi.org/10.1167/iovs.10-5278)
6. Mittra RA, Mieler WF. Drug toxicity of the posterior segment. In: *Retina.* Elsevier; 2013. p. 1532-54. DOI: [10.1016/B978-1-4557-0737-9.00089-8](https://doi.org/10.1016/B978-1-4557-0737-9.00089-8)
7. Geamănu Pancă A, Popa-Cherecheanu A, Marinescu B, Geamănu CD, Voinea LM. Retinal toxicity associated with chronic exposure to hydroxychloroquine and its ocular screening. Review. *J Med Life.* 2014; 7:322-6. PMID: [25408748](https://pubmed.ncbi.nlm.nih.gov/25408748/)
8. Levy GD, Munz SJ, Paschal J, Cohen HB, Pince KJ, Peterson T. Incidence of hydroxychloroquine retinopathy in 1,207 patients in a large multicenter outpatient practice. *Arthritis Rheum.* 1997; 40:1482-6. DOI: [10.1002/art.1780400817](https://doi.org/10.1002/art.1780400817)

9. Mavrikakis I, Sfrikakis PP, Mavrikakis E, Rougas K, Nikolaou A, Kostopoulos C, Mavrikakis M. The incidence of irreversible retinal toxicity in patients treated with hydroxychloroquine: a reappraisal. *Ophthalmology*. 2003; 110:1321-6. DOI: [10.1016/S0161-6420\(03\)00409-3](https://doi.org/10.1016/S0161-6420(03)00409-3)
10. Marmor MF, Kellner U, Lai TYY, Melles RB, Mieler WF. Recommendations on screening for chloroquine and Hydroxychloroquine Retinopathy (2016 revision). *Ophthalmology*. 2016; 123:1386-94. DOI: [10.1016/j.ophtha.2016.01.058](https://doi.org/10.1016/j.ophtha.2016.01.058)
11. Thomas J. Stokkermans; Georgios Trichonas. Chloroquine And Hydroxychloroquine Toxicity. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020-2019 Jun 4.
12. Wetterholm DH, Winter FC. Histopathology of chloroquine retinal toxicity. *Arch Ophthalmol*. 1964; 71:82-7. DOI: [10.1001/archopht.1964.00970010098016](https://doi.org/10.1001/archopht.1964.00970010098016)
13. Rimpelä A-K, Hagström M, Kidron H, Urtti A. Melanin targeting for intracellular drug delivery: Quantification of bound and free drug in retinal pigment epithelial cells. *J Control Release*. 2018; 283:261-8. DOI: [10.1016/j.jconrel.2018.05.034](https://doi.org/10.1016/j.jconrel.2018.05.034)
14. Rimpelä A-K, Schmitt M, Latonen S, Hagström M, Antopolsky M, Manzanares JA, Kidron H, Urtti A. Drug distribution to retinal pigment epithelium: Studies on melanin binding, cellular kinetics, and single photon emission computed tomography/computed tomography imaging. *Mol Pharm*. 2016; 13:2977-86. DOI: [10.1021/acs.molpharmaceut.5b00787](https://doi.org/10.1021/acs.molpharmaceut.5b00787)
15. Bahrpeyma S, Rimpelä A-K, Hagström M, Urtti A. Ocular melanin binding of drugs: in vitro binding studies. *Acta Ophthalmol*. 2019; 97(S263). DOI: [10.1111/j.1755-3768.2019.5366](https://doi.org/10.1111/j.1755-3768.2019.5366)
16. Rosenthal AR, Kolb H, Bergsma D, Huxsoll D, Hopkins JL. Chloroquine retinopathy in the rhesus monkey. *Invest Ophthalmol Vis Sci*. 1978; 17:1158-75.
17. Bernstein H, Zvaifler N, Rubin M, Mansour AM. The ocular deposition of chloroquine. *Invest Ophthalmol*. 1963; 2:384-92.
18. Browning DJ. Pharmacology of Chloroquine and Hydroxychloroquine. In: *Hydroxychloroquine and Chloroquine Retinopathy*. New York, NY: Springer New York; 2014. p. 35-63. DOI: [10.1007/978-1-4939-0597-3_2](https://doi.org/10.1007/978-1-4939-0597-3_2)
19. Gaynes BI, Torczynski E, Varro Z, Grostern R, Perlman J. Retinal toxicity of chloroquine hydrochloride administered by intraperitoneal injection. *J Appl Toxicol*. 2008; 28:895-900. DOI: [10.1002/jat.1353](https://doi.org/10.1002/jat.1353)
20. Hallberg A, Naeser P, Andersson A. Effects of long-term chloroquine exposure on the phospholipid metabolism in retina and pigment epithelium of the mouse. *Acta Ophthalmol*. 1990; 68:125-30. DOI: [10.1111/j.1755-3768.1990.tb01892.x](https://doi.org/10.1111/j.1755-3768.1990.tb01892.x)
21. Ivanina TA, Zueva MV, Lebedeva MN, Bogoslovsky AI, Bunin AJ. Ultrastructural alterations in rat and cat retina and pigment epithelium induced by chloroquine. *Arbeitsphysiologie*. 1983; 220:32-8. DOI: [10.1007/BF02307013](https://doi.org/10.1007/BF02307013)
22. Meier-Ruge W. Experimental investigation of the morphogenesis of chloroquine retinopathy. *Arch Ophthalmol*. 1965; 73:540-4. DOI: [10.1001/archopht.1965.00970030542017](https://doi.org/10.1001/archopht.1965.00970030542017)
23. Al-Bari MAA. Chloroquine analogues in drug discovery: new directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. *J Antimicrob Chemother*. 2015; 70:1608-21. DOI: [10.1093/jac/dkv018](https://doi.org/10.1093/jac/dkv018)

24. Guha S, Coffey EE, Lu W, Lim JC, Beckel JM, Laties AM, Boesze-Battaglia K, Mitchell CH. Approaches for detecting lysosomal alkalization and impaired degradation in fresh and cultured RPE cells: evidence for a role in retinal degenerations. *Exp Eye Res.* 2014; 126:68-76. DOI: [10.1016/j.exer.2014.05.013](https://doi.org/10.1016/j.exer.2014.05.013)
25. Costedoat-Chalumeau N, Dunogu   B, Leroux G, Morel N, Jallouli M, Le Guern V, Piette J, Br  zin AP, Melles RB and Marmor MF. A critical review of the effects of hydroxychloroquine and chloroquine on the eye. *Clin Rev Allergy Immunol.* 2015; 49:317-26. DOI: [10.1007/s12016-015-8469-8](https://doi.org/10.1007/s12016-015-8469-8)
26. Homewood CA, Warhurst DC, Peters W, Baggaley VC. Lysosomes, pH and the anti-malarial action of chloroquine. *Nature.* 1972; 235:50-2. DOI: [10.1038/235050a0](https://doi.org/10.1038/235050a0)
27. Boya P, Kroemer G. Lysosomal membrane permeabilization in cell death. *Oncogene.* 2008; 27:6434-51. DOI: [10.1038/onc.2008.310](https://doi.org/10.1038/onc.2008.310)
28. Green DR, Llambi F. Cell death signaling. *Cold Spring Harb Perspect Biol.* 2015; 7:a006080. DOI: [10.1101/cshperspect.a006080](https://doi.org/10.1101/cshperspect.a006080)
29. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nu  ez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon H-U, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G and Kroemer G. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 2012; 19:107-20. DOI: [10.1038/cdd.2011.96](https://doi.org/10.1038/cdd.2011.96)
30. Cryns V, Yuan J. Proteases to die for. *Genes Dev.* 1998; 12:1551-70. DOI: [10.1101/gad.12.11.1551](https://doi.org/10.1101/gad.12.11.1551)
31. Robertson JD, Orrenius S. Role of mitochondria in toxic cell death. *Toxicology.* 2002; 181-182:491-6. DOI: [10.1016/S0300-483X\(02\)00464-X](https://doi.org/10.1016/S0300-483X(02)00464-X)
32. Cheng AG, Cunningham LL, Rubel EW. Hair cell death in the avian basilar papilla: characterization of the in vitro model and caspase activation. *J Assoc Res Otolaryngol.* 2003; 4:91-105. DOI: [10.1007/s10162-002-3016-8](https://doi.org/10.1007/s10162-002-3016-8)
33. Nicotera TM, Hu BH, Henderson D. The caspase pathway in noise-induced apoptosis of the chinchilla cochlea. *J Assoc Res Otolaryngol.* 2003; 4:466-77. DOI: [10.1007/s10162-002-3038-2](https://doi.org/10.1007/s10162-002-3038-2)
34. Bergsb  ken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol.* 2009; 7:99-109. DOI: [10.1038/nrmicro2070](https://doi.org/10.1038/nrmicro2070)
35. Denes A, Lopez-Castejon G, Brough D. Caspase-1: is IL-1 just the tip of the ICEberg? *Cell Death Dis.* 2012; 3:e338. DOI: [10.1038/cddis.2012.86](https://doi.org/10.1038/cddis.2012.86)
36. Dinarello CA. Interleukin-1  ta, interleukin-18, and the interleukin-1  ta converting enzyme. *Ann N Y Acad Sci.* 1998; 856:1-11. DOI: [10.1111/j.1749-6632.1998.tb08307.x](https://doi.org/10.1111/j.1749-6632.1998.tb08307.x)
37. Fantuzzi G, Dinarello CA. Interleukin-18 and interleukin-1   ta: two cytokine substrates for ICE (caspase-1). *J Clin Immunol.* 1999; 19:1-11. DOI: [10.1023/A:1020506300324](https://doi.org/10.1023/A:1020506300324)
38. Tseng WA, Thein T, Kinnunen K, Lashkari K, Gregory MS, D'Amore PA, Ksander BR. NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2013; 54:110-20. DOI: [10.1167/iovs.12-10655](https://doi.org/10.1167/iovs.12-10655)
39. Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C,

- Wagner G, Hedrick SM, Gerber SA, Lugovskoy A and Yuan J. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol.* 2008; 4:313-21. DOI: [10.1038/nchembio.83](https://doi.org/10.1038/nchembio.83)
40. Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell.* 2008; 135:1311-23. DOI: [10.1016/j.cell.2008.10.044](https://doi.org/10.1016/j.cell.2008.10.044)
41. Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG. Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. *Proc Natl Acad Sci U S A.* 2010; 107:21695-700. DOI: [10.1073/pnas.1009179107](https://doi.org/10.1073/pnas.1009179107)
42. Rosenbaum DM, Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI. Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. *J Neurosci Res.* 2009; NA-NA. DOI: [10.1002/jnr.22314](https://doi.org/10.1002/jnr.22314)
43. Kim LA, Amarnani D, Gnanaguru G, Tseng WA, Vavvas DG, D'Amore PA. Tamoxifen toxicity in cultured retinal pigment epithelial cells is mediated by concurrent regulated cell death mechanisms. *Invest Ophthalmol Vis Sci.* 2014; 55:4747-58. DOI: [10.1167/iovs.13-13662](https://doi.org/10.1167/iovs.13-13662)
44. Xin-Zhao Wang C, Zhang K, Aredo B, Lu H, Ufret-Vincenty RL. Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis. *Exp Eye Res.* 2012; 102:1-9. DOI: [10.1016/j.exer.2012.06.003](https://doi.org/10.1016/j.exer.2012.06.003)
45. Wang J, Iacovelli J, Spencer C, Saint-Geniez M. Direct effect of sodium iodate on neurosensory retina. *Invest Ophthalmol Vis Sci.* 2014; 55:1941-53. DOI: [10.1167/iovs.13-13075](https://doi.org/10.1167/iovs.13-13075)
46. Gnanaguru G, Choi AR, Amarnani D, D'Amore PA. Oxidized lipoprotein uptake through the CD36 receptor activates the NLRP3 inflammasome in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2016; 57:4704-12. DOI: [10.1167/iovs.15-18663](https://doi.org/10.1167/iovs.15-18663)
47. Hobbs HE, Sorsby A, Freedman A. Retinopathy following chloroquine therapy. *Lancet.* 1959; 274:478-80. DOI: [10.1016/S0140-6736\(59\)90604-X](https://doi.org/10.1016/S0140-6736(59)90604-X)
48. Kader DHA, Sayed SSED, El-Ghamrawy TA. Chloroquine-Induced Retinopathy in the Rat. Immunohistochemical and Ultrastructural Study. *J Med Sci (Faisalabad, Pakistan).* 2007; 7:1225-38. DOI: [10.3923/jms.2007.1225.1238](https://doi.org/10.3923/jms.2007.1225.1238)
49. Bhattacharyya B, Chatterjee TK, Ghosh JJ. Effects of chloroquine on lysosomal enzymes, NADPH-induced lipid peroxidation, and antioxidant enzymes of rat retina. *Biochem Pharmacol.* 1983; 32:2965-8. DOI: [10.1016/0006-2952\(83\)90403-3](https://doi.org/10.1016/0006-2952(83)90403-3)
50. Hornung V, Bauernfeind F, Halle A, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol.* 2008; 9: 847-856. DOI: [10.1038/ni.1631](https://doi.org/10.1038/ni.1631)
51. Rajamaki K, Lappalainen J, Oorni K, et al. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One.* 2010; 5: e11765. DOI: [10.1371/journal.pone.0011765](https://doi.org/10.1371/journal.pone.0011765)
52. Tarallo V, Hirano Y, Gelfand BD, et al. DICER1 loss and Alu RNA induce age-related macular degeneration via the NLRP3 inflammasome and MyD88. *Cell.* 2012; 149: 847-859. DOI: [10.1016/j.cell.2012.03.036](https://doi.org/10.1016/j.cell.2012.03.036)